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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (c).

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TITLE OF THE INVENTION (500 characters max)

OPTIMIZED EXPRESSION OF HPV 52 L1 IN YEAST

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Respectfully submitted,

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TITLE OF THE INVENTION

OPTIMIZED EXPRESSION OF HPV 52 L1 IN YEAST

FIELD OF THE INVENTION

5 The present invention relates generally to the prevention and/or therapy of human papillomavirus (HPV) infection. More specifically, the present invention relates to synthetic polynucleotides encoding HPV 52 L1 protein, and to recombinant vectors and hosts comprising said polynucleotides. This invention also relates to HPV 52 virus-like particles (VLPs), wherein the VLPs are produced by expressing recombinant HPV 52 L1 or L1 + L2 in yeast cells and to their use in vaccines
10 and pharmaceutical compositions for preventing and treating HPV infections.

BACKGROUND OF THE INVENTION

 There are more than 80 types of human papillomavirus (HPV), many of which have been associated with a wide variety of biological phenotypes, from benign proliferative warts to malignant
15 carcinomas (for review, *see* McMurray et al., *Int. J. Exp. Pathol.* 82(1): 15-33 (2001)). HPV6 and HPV11 are the types most commonly associated with benign warts, nonmalignant condyloma acuminata and/or low-grade dysplasia of the genital or respiratory mucosa. HPV16 and HPV18 are the high-risk types most frequently associated with *in situ* and invasive carcinomas of the cervix, vagina, vulva and anal canal. More than 90% of cervical carcinomas are associated with infections of HPV16, HPV18 or
20 the less prevalent oncogenic types HPV31, -33, -45, -52 and -58 (Schiffman et al., *J. Natl. Cancer Inst.* 85(12): 958-64 (1993)). The observation that HPV DNA is detected in 90-100% of cervical cancers provides strong epidemiological evidence that HPVs cause cervical carcinoma (*see* Bosch et al., *J. Clin. Pathol.* 55: 244-265 (2002)).

 Papillomaviruses are small (50-60 nm), nonenveloped, icosahedral DNA viruses that
25 encode up to eight early and two late genes. The open reading frames (ORFs) of the viral genomes are designated E1 to E7, and L1 and L2, where "E" denotes early and "L" denotes late. L1 and L2 code for virus capsid proteins, while the E genes are associated with functions such as viral replication and cellular transformation.

 The L1 protein is the major capsid protein and has a molecular weight of 55-60 kDa.
30 The L2 protein is the minor capsid protein. Immunological data suggest that most of the L2 protein is internal to the L1 protein in the viral capsid. Both the L1 and L2 proteins are highly conserved among different papillomaviruses.

 Expression of the L1 protein or a combination of the L1 and L2 proteins in yeast, insect cells, mammalian cells or bacteria leads to self-assembly of virus-like particles (VLPs) (for review, *see*
35 Schiller and Roden, in *Papillomavirus Reviews: Current Research on Papillomaviruses*; Lacey, ed.

Leeds, UK: Leeds Medical Information, pp 101-12 (1996)). VLPs are morphologically similar to authentic virions and are capable of inducing high titres of neutralizing antibodies upon administration into animals or humans. Because VLPs do not contain the potentially oncogenic viral genome, they present a safe alternative to the use of live virus in HPV vaccine development (for review, *see* Schiller and Hidesheim, *J. Clin. Virol.* 19: 67-74 (2000)). For this reason, the L1 and L2 genes have been identified as immunological targets for the development of prophylactic and therapeutic vaccines for HPV infection and disease.

HPV vaccine development and commercialization have been hindered by difficulties associated with obtaining high expression levels of capsid proteins in successfully transformed host organisms, limiting the production of purified protein. Therefore, despite the identification of wild-type nucleotide sequences encoding HPV L1 proteins such as HPV 52 L1 protein, it would be highly desirable to develop a readily renewable source of crude HPV L1 protein that utilizes HPV 52 L1-encoding nucleotide sequences that are optimized for expression in the intended host cell. Additionally, it would be useful to produce large quantities of HPV 52 L1 VLPs having the immunity-conferring properties of the native proteins for use in vaccine development.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by HPV 52 L1 genes. Specifically, the present invention provides polynucleotides encoding HPV 52 L1 protein, wherein the polynucleotides have been codon-optimized for high level expression in a yeast cell. In alternative embodiments of the invention, the nucleotide sequence of the polynucleotide is altered to eliminate transcription termination signals that are recognized by yeast. The present invention further provides HPV 52 virus-like particles (VLPs), wherein said VLPs are produced by expressing recombinant HPV 52 L1 or L1 + L2 in yeast cells, and discloses use of HPV 52 VLPs in immunogenic compositions and vaccines for the prevention and/or treatment of HPV disease and HPV-associated cancer.

The present invention relates to synthetic DNA molecules encoding the HPV 52 L1 protein. The codons of the synthetic molecules are designed so as to use the codons preferred by a yeast cell. In an alternative embodiment of the invention, the nucleotide sequence of the synthetic molecule is altered to eliminate transcription termination signals that are recognized by yeast. The synthetic molecules may be used as a source of HPV 52 L1 protein, which may self-assemble into VLPs. Said VLPs may be used in a VLP-based vaccine.

An exemplary embodiment of the present invention comprises a synthetic nucleic acid molecule which encodes the HPV 52 L1 protein as set forth in SEQ ID NO:2, said nucleic acid molecule comprising a sequence of nucleotides that is codon-optimized for high-level expression in a yeast cell.

Also provided are recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification. In a preferred embodiment of the present invention, the host cell is a yeast cell.

The present invention also relates to a process for expressing an HPV 52 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV 52 L1 protein into a yeast host cell; and (b) culturing the yeast host cell under conditions which allow expression of said HPV 52 L1 protein.

The present invention further relates to a process for expressing an HPV 52 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid molecule encoding an HPV 52 L1 protein into a yeast host cell; wherein the nucleic acid molecule is codon-optimized for optimal expression in the yeast host cell and; (b) culturing the yeast host cell under conditions which allow expression of said HPV 52 L1 protein.

In preferred embodiments, the nucleic acid molecule comprises a sequence of nucleotides as set forth in SEQ ID NO:1 (designated herein "52 L1 R sequence").

This invention also relates to HPV 52 virus-like particles (VLPs) which are produced in yeast cells, methods of producing HPV 52 VLPs, and methods of using HPV 52 VLPs.

In a preferred embodiment of the invention, the yeast is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.

Another aspect of this invention is an HPV 52 VLP, wherein the VLP is produced by recombinant expression of HPV 52 L1 or HPV 52 L1 + L2 in a yeast cell.

Yet another aspect of this invention is an HPV 52 VLP which comprises an HPV 52 L1 protein produced by a codon-optimized HPV 52 L1 gene. In an exemplary embodiment of this aspect of the invention, the codon-optimized HPV 52 L1 gene comprises a sequence of nucleotides as set forth in SEQ ID NO:1.

This invention also provides a method for inducing an immune response in an animal comprising administering HPV 52 virus-like particles to the animal. In a preferred embodiment, the HPV 52 VLPs are produced by a codon-optimized gene.

Yet another aspect of this invention is a method of preventing or treating HPV-associated cervical cancer comprising administering to a mammal a vaccine comprising HPV 52 VLPs. In a preferred embodiment of this aspect of the invention, the HPV 52 VLPs are produced in yeast.

This invention also relates to a vaccine comprising HPV 52 virus-like particles (VLPs), wherein the HPV 52 VLPs are produced in yeast.

In an alternative embodiment of this aspect of the invention, the vaccine further comprises VLPs of at least one additional HPV type. The at least one additional HPV type may be any

HPV type of interest, including any HPV type described in the art or those subsequently identified. In a preferred embodiment, the HPV type is a type that is associated with a clinical phenotype such as warts or cervical cancer. In a further preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45,
 5 HPV51, HPV55, HPV56, HPV58, HPV59, and HPV68.

This invention also relates to pharmaceutical compositions comprising HPV 52 virus-like particles, wherein the HPV 52 VLPs are produced in yeast. Further, this invention relates to pharmaceutical compositions comprising HPV 52 VLPs and VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of:
 10 HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV55, HPV56, HPV58, HPV59, and HPV68.

As used throughout the specification and in the appended claims, the singular forms “a,” “an,” and “the” include the plural reference unless the context clearly dictates otherwise.

15 As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed “enhancers” or
 20 “upstream activating sequences” or inhibiting sequences termed "silencers".

The term “vector” refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmids, viruses (including adenovirus), bacteriophages and cosmids.

The term “cassette” refers to a nucleotide or gene sequence that is to be expressed from a
 25 vector, for example, the nucleotide or gene sequence encoding the HPV 52 L1 protein. In general, a cassette comprises a gene sequence inserted into a vector which, in some embodiments, provides regulatory sequences for expressing the nucleotide or gene sequence. In other embodiments, the nucleotide or gene sequence provides the regulatory sequences for its expression. In further
 30 embodiments, the vector provides some regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. For example, the vector can provide a promoter for transcribing the nucleotide or gene sequence and the nucleotide or gene sequence provides a transcription termination sequence. The regulatory sequences which can be provided by the vector include, but are not limited to, enhancers, transcription termination sequences, splice acceptor and donor sequences, introns, ribosome binding sequences, and poly(A) addition sequences.

The designations “52 L1 wild-type sequence” and “52 L1 wt sequence” refer to the HPV 52 L1 sequence disclosed herein as SEQ ID NO:3. Although the HPV 52 L1 wild-type sequence has been described previously, it is not uncommon to find minor sequence variations between DNAs obtained from clinical isolates. Therefore, a representative HPV 52 L1 wild-type sequence was isolated from clinical samples previously shown to contain HPV 52 DNA (see EXAMPLE 1). The HPV 52 L1 wild-type sequence was used as a reference sequence to compare the codon-optimized HPV 52 L1 sequences disclosed herein (see FIGURE 1).

The designations “HPV 52 L1 R” and “52 L1 R” refer to an exemplary synthetic HPV52 L1 nucleotide sequence (SEQ ID NO:1), disclosed herein, wherein the sequence was rebuilt so that it comprises codons that are preferred for high-level expression by a yeast cell.

The term “effective amount” means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

A “conservative amino acid substitution” refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

The term “mammalian” refers to any mammal, including a human being.

“VLP” or “VLPs” mean(s) virus-like particle or virus-like particles.

“Synthetic” means that the HPV 52 L1 gene was created so that it contains a sequence of nucleotides that is not the same as the sequence of nucleotides present in the designated naturally occurring wild-type HPV 52 L1 gene (52 L1 wt, SEQ ID NO:3). As stated above, synthetic molecules are provided herein comprising a sequence of nucleotides comprising codons that are preferred for expression by yeast cells. The synthetic molecules provided herein encode the same amino acid sequences as the wild-type HPV 52 L1 gene (SEQ ID NO:2).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a sequence alignment comparing nucleotides that were altered in the synthetic HPV 52 L1 gene of the present invention (SEQ ID NO:1, indicated as “52 L1 R”) (See EXAMPLE 2). The reference sequence is the 52 L1 wild-type sequence (SEQ ID NO:3, indicated as “52 L1 wt”; see EXAMPLE 1). Altered nucleotides are indicated at their corresponding location. Nucleotide number is contained within the parentheses. Identical nucleotides in the 52 L1 rebuilt sequence are indicated with dots.

FIGURE 2 shows the rebuilt synthetic HPV 52 L1 double-stranded nucleic acid (SEQ ID NOs:1 and 7) and single-code amino acid sequence (SEQ ID NO:2). Nucleotide number is indicated to the left.

FIGURE 3 shows a Northern blot of HPV 52 L1 wt and HPV 52 L1 R transcripts (see EXAMPLE 4). The blot was probed with a mixture of DNA probes generated against both the 52 L1 wt and the 52 L1 R sequences. The arrow on right indicates the predicted position of a full-length 52 L1 transcript. No transcripts of any length were detected in the 5 and 10 µg lanes of 52 L1 wt RNA. Full-length transcripts are apparent in the 52 L1 R, in both the 5 and 10 µg lanes.

FIGURE 4 shows a Western Blot of HPV 52 L1 wt (52 wt), and 52 L1 R (52R) proteins. HPV 16 L1 was included as a reference (16). Ten, five and two and one-half micrograms of total yeast protein extract were denatured and applied to a 10% SDS-PAGE gel. The protein was Western transferred. HPV 52 L1 protein was detected on the resulting blot using a yeast-absorbed anti-trpE-HPV 31 L1 goat polyclonal antiserum which cross-reacts with HPV 52 L1 and HPV 16 L1. Molecular weight markers are indicated in kDa on the left. The arrow indicates the position of the ~ 55 kDa HPV 52 L1 protein.

FIGURE 5 shows a representative sample of HPV 52 VLPs composed of HPV 52 L1 R protein molecules, described herein, as visualized by transmission electron microscopy (see EXAMPLE 7). The diameter of the spherical particles in this crude sample ranged from between 40 and 70 nm with some particles displaying a regular array of capsomers. The bar represents approximately 0.1 µm.

DETAILED DESCRIPTION OF THE INVENTION

The majority of cervical carcinomas are associated with infections of specific oncogenic types of human papillomavirus (HPV). The present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by genes of oncogenic HPV types.

Specifically, the present invention provides polynucleotides encoding HPV 52 L1, wherein the polynucleotides are codon-optimized for high-level expression in yeast. The present invention also provides HPV52 virus-like particles (VLPs), which are produced in yeast, and discloses use of said polynucleotides and VLPs in immunogenic compositions and vaccines for the prevention and/or treatment of HPV-associated cancer.

A wild-type HPV52 L1 nucleotide sequence has been reported (Genbank Accession # NC 001592). The present invention provides synthetic DNA molecules encoding the HPV 52 L1 protein. In one aspect of the invention, the synthetic molecules comprise a sequence of codons, wherein at least some of the codons have been altered to use the codons preferred by a yeast cell for high-level expression. In an alternative aspect of the invention, the nucleotide sequence of the synthetic molecule is altered to eliminate transcription termination signals that are recognized by yeast. The synthetic

molecules may be used as a coding sequence for expression of HPV 52 L1 protein, which may self-assemble into VLPs. Said VLPs may be used in a VLP-based vaccine to provide effective immunoprophylaxis against papillomavirus infection through neutralizing antibody and cell-mediated immunity. Such VLP-based vaccines may also be useful for treatment of already established HPV infections.

Expression of HPV VLPs in yeast cells offers the advantages of being cost-effective and easily adapted to large-scale growth in fermenters. In addition, the yeast genome can be readily altered to ensure selection of recombinant, transformed yeast with increased growth and expression potential. However, many HPV L1 proteins, including HPV 52 L1 are expressed at levels in yeast cells which are lower than what is desirable for commercial scale-up (see EXAMPLE 2).

Accordingly, the present invention relates to HPV 52 L1 gene sequences that are "optimized" for high-level expression in a yeast cellular environment.

A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon, a phenomenon known as codon redundancy. For reasons not completely understood, alternative codons are not uniformly present in the endogenous DNA of differing types of cells. Indeed, there appears to exist a variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG. Exhaustive analysis of genome codon use frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally believed that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For example, it is likely that a gene rich in TTA codons will be poorly expressed in *E. coli*, whereas a CTG rich gene will probably be highly expressed in this host. Similarly, a preferred codon for expression of a leucine-rich polypeptide in yeast host cells would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms--a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide an optimal form of foreign genetic material for practice of recombinant protein expression. Thus, one aspect of this invention is an HPV 52 L1 gene that is codon-optimized for high-level expression in a yeast cell. In a preferred embodiment of this invention, it has

been found that the use of alternative codons encoding the same protein sequence may remove the constraints on expression of HPV 52 L1 proteins by yeast cells.

In accordance with this invention, HPV 52 L1 gene segments were converted to sequences having identical translated sequences but with alternative codon usage as described by Sharp and Cowe (Synonymous Codon Usage in *Saccharomyces cerevisiae*. *Yeast* 7: 657-678 (1991)), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed yeast genes and replacing them with optimal codons for high expression in yeast cells. The new gene sequence is then inspected for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, high GC content, presence of transcription termination signals that are recognized by yeast, etc.). Undesirable sequences are eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

The methods described above were used to create synthetic gene segments for HPV 52 L1, resulting in a gene comprising codons optimized for high-level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for use in HPV vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

Accordingly, the present invention relates to a synthetic polynucleotide comprising a sequence of nucleotides encoding an HPV 52 L1 protein, or a biologically active fragment or mutant form of an HPV 52 L1 protein, the polynucleotide sequence comprising codons optimized for expression in a yeast host cell. Said mutant forms of the HPV 52 L1 protein include, but are not limited to: conservative amino acid substitutions, amino-terminal truncations, carboxy-terminal truncations, deletions, or additions. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the immunological properties of the HPV 52 L1 protein as set forth in SEQ ID NO:2. The synthetic polynucleotides of the present invention encode mRNA molecules that express a functional HPV 52 L1 protein so as to be useful in the development of a therapeutic or prophylactic HPV vaccine.

One aspect of this invention is a codon-optimized nucleic acid molecule which encodes the HPV 52 L1 protein as set forth in SEQ ID NO:2, said nucleic acid molecule comprising a sequence of nucleotides that are codon-optimized for high-level expression in a yeast cell. In a preferred embodiment of this aspect of the invention, the nucleic acid molecule comprises a sequence of nucleotides as set forth in SEQ ID NO:1.

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification. In a preferred embodiment of this invention, the host cell is a yeast host cell.

The synthetic HPV 52 L1 DNA, functional equivalents thereof, and fragments thereof, constructed through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements. Said expression vector may be transferred into prokaryotic or eukaryotic host cells to produce recombinant HPV 52 L1 protein. Techniques for such manipulations are fully described in the art (Sambrook et al. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989); *Current Protocols in Molecular Biology*, Ausubel et al., Green Pub. Associates and Wiley-Interscience, New York (1988); *Yeast Genetics: A Laboratory Course Manual*, Rose et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1990), which are hereby incorporated by reference in their entirety).

Thus, the present invention relates to a process for expressing an HPV 52 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV 52 L1 protein into a yeast host cell; and (b) culturing the yeast host cell under conditions which allow expression of said HPV 52 L1 protein.

The present invention further relates to a process for expressing an HPV 52 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV 52 L1 protein into a yeast host cell; wherein the nucleic acid molecule is codon-optimized for optimal expression in the yeast host cell and; (b) culturing the yeast host cell under conditions which allow expression of said HPV 52 L1 protein.

This invention further relates to a process for expressing an HPV 52 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid as set forth in SEQ ID NO:1 into a yeast host cell; and, (b) culturing the yeast host cell under conditions which allow expression of said HPV 52 L1 protein.

The synthetic genes of the present invention can be assembled into an expression cassette that comprises sequences designed to provide efficient expression of the HPV 52 L1 protein in the host cell. The cassette preferably contains the synthetic gene, with related transcriptional and translational control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the *S. cerevisiae GAL1* promoter, although those skilled in the art will recognize that any of a number of other known yeast promoters such as the *GAL10*, *GAL7*, *ADH1*, *TDH3* or *PGK* promoters, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the *S. cerevisiae ADH1* terminator, although other known transcriptional terminators may also be used. The combination of *GAL1* promoter – *ADH1* terminator is particularly preferred.

Another aspect of this invention is an HPV 52 virus-like particle (VLP) produced by recombinantly expressing the HPV 52 L1 or L1 + L2 genes in a yeast cell, methods of producing HPV 52 VLPs, and methods of using HPV 52 VLPs. VLPs can self-assemble when L1, the major capsid protein of human and animal papillomaviruses, is expressed in yeast, insect cells, mammalian cells or bacteria (for review, see Schiller and Roden, in *Papillomavirus Reviews: Current Research on Papillomaviruses*; Lacey, ed. Leeds, UK: Leeds Medical Information, pp 101-12 (1996)). Morphologically indistinct HPV VLPs can also be produced by expressing a combination of the L1 and L2 capsid proteins. VLPs are composed of 72 pentamers of L1 in a T=7 icosahedral structure (Baker et al., *Biophys. J.* 60(6): 1445-56 (1991)).

VLPs are morphologically similar to authentic virions and are capable of inducing high titres of neutralizing antibodies upon administration into an animal. Immunization of rabbits (Breitburd et al., *J. Virol.* 69(6): 3959-63 (1995)) and dogs (Suzich et al., *Proc. Natl. Acad. Sci. USA* 92(25): 11553-57 (1995)) with VLPs was shown to both induce neutralizing antibodies and protect against experimental papillomavirus infection. Additionally, immunization of adult women with HPV 16 VLPs was shown to protect against HPV 16 infection and HPV 16 cervical intraepithelial neoplasia (Koutsky et al. *N. Engl. J. Med.* 347: 1645-51 (2002)). Because VLPs do not contain the potentially oncogenic viral genome and can self-assemble when expressed from a single gene, they present a safe alternative to the use of live virus in HPV vaccine development (for review, see Schiller and Hidesheim, *J. Clin. Virol.* 19: 67-74 (2000)).

Thus, the present invention relates to virus-like particles comprised of recombinant L1 protein or recombinant L1 + L2 proteins of HPV 52, wherein the recombinant protein is expressed in a yeast cell.

As stated above, in a preferred embodiment of the invention, the HPV 52 VLPs are produced in yeast. In a further preferred embodiment, the yeast is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyvermyces fragilis*, *Kluveromyces lactis*, and *Schizosaccharomyces pombe*.

Another aspect of this invention is an HPV 52 VLP which comprises an HPV 52 L1 protein produced by a codon-optimized HPV 52 L1 gene. In a preferred embodiment of this aspect of the invention, the codon-optimized HPV 52 L1 gene comprises a sequence of nucleotides as set forth in SEQ ID NO:1.

Yet another aspect of this invention is a method of producing HPV 52 VLPs, comprising: (a) transforming yeast with a recombinant DNA molecule encoding HPV 52 L1 protein or HPV 52 L1 + L2 proteins; (b) cultivating the transformed yeast under conditions that permit expression of the recombinant DNA molecule to produce the recombinant HPV 52 protein; and (c) isolating the recombinant HPV 52 protein to produce HPV52 VLPs.

In a preferred embodiment of this aspect of the invention, the yeast is transformed with a codon-optimized HPV 52 L1 gene to produce HPV 52 VLPs. In a particularly preferred embodiment, the codon-optimized HPV 52 L1 gene comprises a sequence of nucleotides as set forth in SEQ ID NO:1.

This invention also provides a method for inducing an immune response in an animal comprising administering HPV 52 virus-like particles to the animal. In a preferred embodiment, the HPV 52 VLPs are produced by recombinantly expressing a codon-optimized gene encoding HPV 52 L1 or HPV 52 L1 + L2.

Yet another aspect of this invention is a method of preventing and/or treating HPV-associated cervical cancer comprising administering to a mammal a vaccine comprising HPV 52 VLPs.

In a preferred embodiment of this aspect of the invention, the HPV 52 VLPs are produced in yeast.

This invention also relates to a vaccine comprising HPV 52 virus-like particles (VLPs).

In an alternative embodiment of this aspect of the invention, the vaccine further comprises VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 55, HPV 56, HPV 58, HPV 59, and HPV 68.

In a preferred embodiment of this aspect of the invention, the vaccine further comprises HPV 16 VLPs.

In another preferred embodiment of the invention, the vaccine further comprises HPV 16 VLPs and HPV 18 VLPs.

In yet another preferred embodiment of the invention, the vaccine further comprises HPV 6 VLPs, HPV 11 VLPs, HPV 16 VLPs and HPV 18 VLPs.

This invention also relates to pharmaceutical compositions comprising HPV 52 virus-like particles. Further, this invention relates to pharmaceutical compositions comprising HPV 52 VLPs and VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 55, HPV 56, HPV 58, HPV 59, and HPV 68.

Vaccine compositions of the present invention may be used alone at appropriate dosages which allow for optimal inhibition of HPV 52 infection with minimal potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The amount of virus-like particles to be introduced into a vaccine recipient will depend on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 10 µg to 100 µg, and preferably about 20 µg to 60 µg of VLPs is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations may be provided.

Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration with adjuvants such as alum or Merck alum adjuvant, concurrently with or subsequent to parenteral introduction of the vaccine of this invention is also advantageous.

5 All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

10 Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

15 EXAMPLE 1

Determination of a Representative HPV 52 L1 Sequence

The HPV 52 L1 sequence has been described previously (Genbank Accession # NC 001592). It is not uncommon, however, to find minor sequence variations between DNAs obtained from clinical isolates. To determine a representative HPV 52 L1 wild-type sequence, DNA was isolated from
20 three clinical samples previously shown to contain HPV 52 DNA. HPV 52 L1 sequences were amplified in a polymerase chain reaction (PCR) using *Taq* DNA polymerase and the following primers: 5' L1 5' – A T G T C C G T G T G G C G G C C T A G T – 3' (SEQ ID NO:4) and 3' 52 Bgl II 5' – G A G A T C T C A A T T A C A C A A A G T G – 3' (SEQ ID NO:5). The amplified products were electrophoresed on agarose gels and visualized by ethidium bromide staining. The ~ 1500 bp L1 bands were excised and
25 DNA was purified using GeneClean Spin Kit (Q-Bio Gene, Carlsbad, CA). The DNA was then ligated to the TA cloning vector, pCR2.1 (Invitrogen). TOP10F' *E. coli* cells were transformed with the ligation mixture and plated on LB agar with kanamycin plus IPTG and X-gal for blue/white colony selection. The plates were inverted and incubated for 16 hours at 37°C.

Colony PCR was performed on five white colonies originating from each of the three
30 clinical isolates amplified. 5' L1 and 3' 52 Bgl II primers were used in a two-step PCR in which the first step comprised 10 cycles of 96°C for 15 seconds (denaturing), 55°C for 30 seconds (annealing) and 68°C for 2 minutes (extension), and the second step comprised 35 cycles of an essentially similar program, except the annealing step was performed at 50°C for 30 seconds. PCR products were electrophoresed on

agarose gels and visualized by ethidium bromide staining. Several colonies from each clinical isolate contained amplified products with ~1500 bp bands. The colonies were cultured in LB medium with kanamycin, shaking at 37°C for 16 hours. Minipreps were performed to extract the plasmid DNAs, which were digested with restriction endonucleases to demonstrate the presence of the L1 gene in the plasmid.

5 The resulting restriction fragments were viewed by agarose gel electrophoresis and ethidium bromide staining.

DNA sequencing was performed on plasmids containing cloned L1 inserts from each of the three clinical isolates. DNA and translated amino acid sequences were compared with one another and the previously published Genbank HPV 52 L1 sequences. Sequence analysis of the three clinical

10 isolates revealed that no sequence was identical to the Genbank sequence (Accession No. NC 001592). The pCR2.1 HPV 52L1 clone #2C was chosen to be the representative HPV 52 L1 sequence and is referred to herein as the “52 L1 wild-type sequence” (SEQ ID NO:3, *see* FIGURE 1). The sequence chosen as 52 L1 wild-type (wt) contained one point mutation when compared to the Genbank sequence, which consisted of a silent mutation at nucleotide 1308 (adenine → guanine). The amino acid sequence

15 of the HPV 52 L1 wt sequence was identical to the 52 L1 Genbank sequence.

The HPV 52 L1 wild-type sequence was amplified using the 5' L1 Bgl II primer (5' – G A G A T C T C A C A A A C A A A A T G T C C G T G T G G C – 3' (SEQ ID NO:6)) and the 3' 52 Bgl II primer described above to add *Bgl* II extensions. PCR was performed using *Taq* polymerase. The PCR product was electrophoresed on an agarose gel and visualized by ethidium bromide staining. The ~

20 1500 bp band was excised and DNA was purified using the GeneClean Spin kit (Q-Bio Gene, Carlsbad, CA). The PCR product was then ligated to the pCR2.1 vector and TOP10F' cells were transformed with the ligation mixture. White colonies were cultured in LB medium with kanamycin, shaking at 37°C for 16 hours. Minipreps were performed to extract the plasmid DNA. The HPV 52 L1 gene was released from the vector sequences with *Bgl* II restriction endonuclease digestions. The digested DNA was

25 subjected to agarose gel electrophoresis and viewed by ethidium bromide staining. The L1 band was purified using the GeneClean kit and ligated to a dephosphorylated, *Bam*HI-digested pGAL110 vector. TOP10F' *E.coli* cells were transformed with the ligation mixture. To screen for the HPV 52 L1 insert in the correct orientation, plasmid DNA from the colonies was PCR-amplified. DNA sequencing was conducted to confirm the sequence and orientation of the inserts. The selected clone was named

30 pGAL110-HPV 52L1 #5. Maxiprep DNA from the selected clone was prepared. *Saccharomyces cerevisiae* cells were made competent by spheroplasting with glucylase and transformed with pGAL110-HPV 52L1 #5. The yeast transformation mixture was plated in Leu⁻ sorbitol top-agar on Leu⁻ sorbitol plates and incubated inverted for 3-5 days at 30°C. Colonies were picked and streaked for isolation on Leu⁻ sorbitol plates. Isolated colonies were subsequently grown in 5 ml of 5 X Leu⁻ Ade⁻ sorbitol with

1.6% glucose and 4% galactose in rotating tube cultures at 30 °C to induce HPV 52 L1 transcription and protein expression.

EXAMPLE 2

5 Yeast codon optimization

Yeast-preferred codons have been described (Sharp, Paul M and Cowe, Elizabeth. Synonymous Codon Usage in *Saccharomyces cerevisiae* YEAST 7: 657-678 (1991)). Expression of the HPV 52 L1 wt protein was detectable; however, the level of transcription was very low and not detectable by Northern blot. It was postulated that pre-mature transcription termination may be responsible for the
 10 low expression levels of the HPV 52 L1 gene. To increase transcription of this gene and ensure full-length transcripts would be produced, the HPV 52 L1 gene was rebuilt utilizing yeast-preferred codons. The sequence was inspected for the presence of yeast transcription termination signals that are recognized by yeast, and these sequences were eliminated by substitution with alternative codons, while preserving the same amino acid sequence. The rebuilt HPV 52 L1 sequence, which comprises yeast codon-
 15 optimized sequences, contained 379 nucleotide alterations compared to the HPV 52 L1 wt sequence. The resulting sequence is referred to herein as “52 L1 R” (R = rebuild, see FIGURE 1). The nucleotide alterations between the 52 L1 wt (SEQ ID NO:3) and 52 L1 R (SEQ ID NO:1) sequences are shown in FIGURE 1. The translated amino acid sequence of 58 L1 R was not altered (SEQ ID NO:2, see FIGURE 2). The rebuilt sequence provides increased HPV 52 L1 protein expression, which is a significant
 20 advance over the wild-type for use in vaccine development.

The strategy employed to produce the optimized gene was to design long overlapping sense and antisense oligomers that span the gene, substituting nucleotides with yeast-preferred codon sequences while maintaining the amino acid sequence. These oligomers were used in place of template DNA in a PCR reaction with *Pfu* DNA polymerase. Additional amplification primers were designed and
 25 used to amplify the rebuilt sequences from template oligomers.

The optimal conditions for amplification were section-specific; however, most reactions employed a program resembling 94°C for 5 minutes (denaturing) followed by 25 cycles of 95°C for 30 sec (denaturing), 50-55°C for 30 sec (annealing), 72°C for 1.5 minute (extension), followed by a 72°C for 7 minute final extension and 4°C hold. PCR products were examined by agarose gel electrophoresis.
 30 Bands of the appropriate size were excised and the DNA was purified from the gel slice. The amplified fragments were then used as templates to assemble the 1512 nt rebuilt HPV 52 L1 gene.

Following rebuild, the 1512 nt band was gel purified, and ligated to pCR4 Blunt vector (Invitrogen, Carlsbad, CA). Following ligation, competent *E. coli* TOP10 cells were transformed with the

ligation mixture. Colonies were grown in 4 ml LB with ampicillin and plasmid DNA was extracted from the colonies by miniprep techniques. The plasmid DNA was sequenced to confirm the presence of the desired HPV 52 L1 rebuild changes. To add *Bam*HI extensions to both ends, the 52 L1 R (rebuild) was re-amplified from pCR4Blunt-52 L1 R. The amplified fragment was cloned as above and the resulting
 5 plasmid DNA was sequenced. The plasmid, pCR4 Blunt-52 L1 R (Bam) was digested with *Bam*HI and the resulting DNA fragment inserts were electrophoresed on an agarose gel. The ~1530 bp HPV 52 L1 R (Bam) fragment was gel purified and ligated to *Bam*HI-digested pGAL110. TOP10F' *E.coli* (Invitrogen) cells were transformed with the ligation mixture.

The resulting colonies were screened by PCR for the HPV 52 L1 R insert in the correct
 10 orientation. Sequence and orientation were confirmed by DNA sequencing. Maxiprep plasmid DNA was prepared. *S. cerevisiae* cells were made competent by spheroplasting and transformed. The yeast transformation was plated in Leu⁻ sorbitol top-agar on Leu⁻ sorbitol agar plates and incubated inverted for 7 days. Colonies were picked and streaked for clonal isolation on Leu⁻ sorbitol agar plates. Isolated colonies were subsequently grown in 5 ml of 5 X Leu⁻ Ade⁻ sorbitol with 1.6% glucose and 4% galactose
 15 in rotating tube cultures at 30°C to induce L1 transcription and protein expression. After 48 and/or 72 hours, a culture volume equivalent to an OD₆₀₀ = 10 was pelleted, the supernatant was removed and the pellets were frozen and stored -70°C.

EXAMPLE 3

20 RNA preparation

Cell pellets of transformed yeast induced to express HPV 52 L1 by galactose induction were thawed on ice, suspended in 0.8 ml of Trizol reagent (Life Technologies, Gibco BRL) and incubated at room temperature for 5 minutes. One fifth volume of chloroform was added to the vial. It was then shaken vigorously for 15 seconds to mix and incubated at room temperature for 3 minutes. After a 5
 25 minute centrifugation at 13 k rpms, the upper phase was collected and transferred to a new vial. 0.4 ml isopropanol was added to the vial. The mixture was incubated at room temperature for 10 minutes. To pellet the RNA, centrifugation was performed at 13 k rpms for 10 minutes. The supernatant was decanted, the RNA pellet washed with 75% EtOH and the centrifugation step was repeated. The supernatant was decanted and the RNA pellet was allowed to air dry for 15 minutes followed by
 30 suspension in RNase-free water. Spectrophotometry was performed to determine the concentration of RNA in the sample using the assumption that an A₂₆₀ reading of 1 = 40 µg/ml RNA when the A_{260/280} is 1.7-2.0.

EXAMPLE 4

Northern Blot Analysis

A 1.1% agarose formaldehyde gel was cast. Five and ten micrograms of RNA were combined with denaturing buffer (final concentrations: 6% formaldehyde, 50% formamide and 0.1 x MOPS) and heated to 65°C for 10 minutes. A one-tenth volume of gel loading buffer was added and the sample was loaded onto the gel. Electrophoresis was performed at 75 volts in 1 x MOPS buffer for ~ 3 hours. The gel was washed for 60 minutes in 10 x SSC.

The RNA was transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, Piscataway, NJ) by capillary action over 16 hours in 10 x SSC. The RNA was then fixed to the nylon membrane by cross-linking using the Stratagene UV Stratalinker auto crosslink function (Stratagene, LA Jolla, CA). After fixing, the nylon membrane was allowed to air dry.

The Roche DIG High Prime DNA Labeling and Detection Kit I (Hoffmann-La Roche Ltd., Basel, Switzerland) was used to label 52 L1 wt and 52 L1 R DNA sequences with DIG to be used as probes to detect 52 L1 wt and 52 L1 R RNA on the Northern blot. The pre-hybridization, hybridization, and immunological development using an anti-DIG alkaline phosphatase-conjugated antibody were performed per the manufactures recommendations. Briefly, the blot was pre-hybridized at 37°C for 30 minutes with gentle shaking. The probe was denatured by heating to 95°C for 5 minutes and subsequent quenching on ice. The probe was added to the hybridization solution and applied to the membrane for 4 hours at 44.6°C with gentle shaking. The hybridization solution was then removed and the blot was washed 2 x for 5 minutes in 2 x SSC with 0.1% SDS at room temperature, followed by an additional wash at 65°C with 0.5 x SSC and 0.1% SDS. The blot was then blocked for 30 minutes and anti-DIG alkaline phosphatase-conjugated antibody was applied at a 1:5000 dilution for 30 minutes. The blot was washed and the presence of probe-bound RNA was determined by NBT/BCIP substrate detection of the alkaline phosphatase conjugated anti-DIG bound antibody.

Initial analysis of yeast expressing HPV 52 L1 wt suggested that HPV 52 L1 protein was expressed; however, the level was low. Northern blot analysis of RNA from yeast extracts of cultures induced to express HPV 52 L1 wt did not reveal any detectable HPV 52 L1 RNA. Since some protein of the appropriate size was detected, it was clear that some full-length RNA transcripts were made. The HPV 52 L1 gene was rebuilt with yeast-preferred codon sequences and was engineered to omit any possible premature transcription termination sites to ensure robust transcription. Northern blot analysis of the HPV 52 L1 R transcript revealed that full-length transcripts were generated and detectable by Northern blot analysis (FIGURE 3).

EXAMPLE 5

HPV 52 L1 Protein Expression

Frozen yeast cell pellets of galactose-induced cultures equivalent to OD₆₀₀= 10 were thawed on ice and suspended in 300 µl of PC buffer (100 mM Na₂HPO₄ and 0.5 M NaCl, pH 7.0) with 2mM PMSF. Acid-washed 0.5mm glass beads were added at a concentration of ~ 0.5g/tube. The tubes were vortexed for 3 cycles of 5 minutes at 4°C with a 1 minute break. 7.5 µl of 20% TritonX100 was added and the vortex step was repeated for 5 minutes at 4°C. The tubes were placed on ice for 15 minutes, followed by centrifugation for 10 minutes at 4°C. The supernatant was transferred to a sterile microcentrifuge tube, which was labeled as total yeast protein extract, dated, and stored at -70°C.

EXAMPLE 6

Western Blot Analysis

Total yeast protein extract from twenty isolated yeast colonies for each HPV 52 L1 construct were analyzed by Western blot to confirm expression of HPV 52 L1 protein after galactose induction.

Ten, five, and two and one-half micrograms of total yeast protein extract were combined with SDS-PAGE loading buffer and heated to 95°C for 10 minutes. The HPV 16 L1 protein, which is approximately 55 kDa, was included as a positive control, along with HPV L1-free total yeast protein extract as a negative control (data not shown). The proteins were loaded onto a 10% SDS-PAGE gel and electrophoresed in Tris-Glycine buffer. After protein separation, the proteins were Western-transferred from the gel to nitrocellulose and the resulting blot was blocked in 1 x diluent buffer (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 hour at room temperature with rocking. The blot was washed three times and yeast absorbed goat anti-trpE-HPV 31 L1 serum, which cross-reacts with HPV 16 and HPV 52 L1 proteins, was applied at room temperature for 16 hours. The blot was then washed three times and incubated with a 1:2500 dilution of anti-goat-HRP conjugated antibody for 1 hr. The blot was again washed three times and NBT/BCIP detection substrate was applied (Kirkegaard and Perry Laboratories). Immunoreactive proteins were detected as purple bands on the blot.

In all cases, the HPV 52 L1 protein was detected as a distinct immunoreactive band on the nitrocellulose corresponding to approximately 55 kDa. (FIGURE 4) The intensity of the HPV 52 L1 R band (2.5 µg lane) appeared to be significantly greater than the HPV 52 L1 wt band (10 µg). It was

clear that upon rebuilding, the expression level of codon-optimized HPV 52 L1 R increased more than four-fold, which is the limit of direct comparison on the Western blot.

EXAMPLE 7

5 Transmission Electron Microscopy

To demonstrate that the 52 L1 protein was in fact self-assembling to form pentameric-L1 capsomers, which in turn self-assemble into virus-like particles, a partially purified HPV 52 L1 R protein extract was subjected to transmission electron microscopy (TEM).

10 Yeast were grown under small scale fermentation and pelleted. The resulting pellets were subjected to purification treatments. Pellet and clarified yeast extracts were analyzed by immunoblot to demonstrate HPV 52 L1 protein expression and retention throughout the purification procedure. Clarified yeast extracts were then subjected to centrifugation over a 45%-sucrose cushion and the resulting pellet was suspended in buffer for analysis of HPV 52 L1 VLPs by TEM.

15 A representative sample of the HPV 52 L1 R VLPs produced is shown in FIGURE 5. The diameter of the spherical particles in this crude sample ranged from between 40 and 70 nm with some particles displaying a regular array of capsomers.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a sequence of nucleotides that encodes an HPV52 L1 protein as set forth in SEQ ID NO:2, the nucleic acid sequence being codon-optimized for high-level expression in a yeast cell.
2. A vector comprising the nucleic acid molecule of claim 1.
3. A host cell comprising the vector of claim 2.
4. The host cell of claim 3, wherein the host cell is a yeast cell.
5. The host cell of claim 4, wherein the yeast cell is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.
6. The host cell of claim 4, wherein the host cell is *Saccharomyces cerevisiae*.
7. The nucleic acid molecule of claim 1, wherein the sequence of nucleotides comprises a sequence of nucleotides as set forth in SEQ ID NO:1.
8. Virus-like particles (VLPs) comprised of recombinant L1 protein or recombinant L1 + L2 proteins of HPV52, wherein the recombinant L1 protein or the recombinant L1 + L2 proteins are produced in yeast.
9. The VLPs of claim 8, wherein the recombinant L1 protein or recombinant L1 + L2 proteins are encoded by a codon-optimized HPV52 L1 nucleic acid molecule.
10. The VLPs of claim 9, wherein the codon-optimized nucleic acid molecule comprises a sequence of nucleotides as set forth in SEQ ID NO:1.
11. A method of producing the VLPs of Claim 9, comprising:
 - (a) transforming yeast with a codon-optimized DNA molecule encoding HPV52 L1 protein or HPV52 L1 + L2 proteins;

- (b) cultivating the transformed yeast under conditions that permit expression of the codon-optimized DNA molecule to produce a recombinant papillomavirus protein; and
- (c) isolating the recombinant papillomavirus protein to produce the VLPs of Claim 9.

12. A vaccine comprising the VLPs of Claim 9.

13. Pharmaceutical compositions comprising the VLPs of claim 9.

14. A method of preventing HPV infection comprising administering the vaccine of Claim 12 to a mammal.

15. A method for inducing an immune response in an animal comprising administering the VLPs of Claim 11 to an animal.

16. The virus-like particles of Claim 9 wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.

17. The virus-like particles of claim 16, wherein the yeast is *Saccharomyces cerevisiae*.

18. The vaccine of claim 12, further comprising VLPs of at least one additional HPV type.

19. The vaccine of claim 18 wherein the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV55, HPV56, HPV58, HPV59, and HPV68.

20. The vaccine of claim 19, wherein the at least one HPV type comprises HPV16.

21. The vaccine of claim 20, further comprising HPV18 VLPs.

22. The vaccine of claim 21, further comprising HPV6 VLPs and HPV11 VLPs.

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- 23. The vaccine of claim 22, further comprising HPV31 VLPs.
- 24. The vaccine of claim 21, further comprising HPV31 VLPs.
- 25. The vaccine of claim 23, further comprising HPV45 VLPs.
- 26. The vaccine of claim 24, further comprising HPV45 VLPs.
- 27. The vaccine of claim 26, further comprising HPV58 VLPs.
- 28. The vaccine of claim 25, further comprising HPV58 VLPs.

ABSTRACT OF THE DISCLOSURE

Synthetic DNA molecules encoding the HPV 52 L1 protein are provided. Specifically, the present invention provides polynucleotides encoding HPV 52 L1 protein, wherein said polynucleotides are codon-optimized for high level expression in a yeast cell. In alternative embodiments of the invention, the nucleotide sequence of the synthetic molecule is altered to eliminate transcription termination signals that are recognized by yeast. The synthetic molecules may be used to produce HPV 52 virus-like particles (VLPs), and to produce vaccines and pharmaceutical compositions comprising the HPV 52 VLPs. The vaccines of the present invention provide effective immunoprophylaxis against papillomavirus infection through neutralizing antibody and cell-mediated immunity and may also be useful for treatment of existing HPV infections.

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FIGURE 1. HPV 52 L1 Nucleotide Sequence Alignment

52 L1 wt	(1)	ATGTCCGTGTGGCGGCCTAGTGAGGCCACTGTGTACCTGCCTCCTGTACC
52 L1 R	C...A.A..ATCC..A..T.....C...T...A..A..T..
52 L1 wt	(51)	TGTCTCTAAGGTTGTAAGCACTGATGAGTATGTGTCTCGCACAAGCATCT
52 L1 R		A.....CTCT..C..C..A..C..C..CA.A..CTC.....
52 L1 wt	(101)	ATTATTATGCAGGCAGTTCTCGATTACTAACAGTAGGACATCCCTATTTT
52 L1 R		.C..C..C..T..TTCC...A....GT.G..T..C..T..C..A..C..C
52 L1 wt	(151)	TCTATTAAAAACACCAGTAGTGGTAATGGTAAAAAAGTTTTAGTTCCCAA
52 L1 R	C..G.....TCCTCC.....C.....G..G..C..G.....A..
52 L1 wt	(201)	GGTGTCTGGCCTGCAATACAGGGTATTTAGAAATTAAATTGCCGGACCCTA
52 L1 R		...C.....TT.....A..C..C.....C..G.....A.....A.
52 L1 wt	(251)	ATAAATTTGGTTTTCCAGATACATCTTTTTATAACCCAGAAACCCAAAGG
52 L1 R		.C..G..C.....C.....C..TAG...C..C.....T.....A
52 L1 wt	(301)	TTGGTGTGGGCCTGTACAGGCTTGGAATTGGTAGGGGACAGCCTTTAGG
52 L1 R	C.....T.....T..T.....C.....A..T..A..A..G..
52 L1 wt	(351)	TGTGGGTATTAGTGGGCATCCTTTATTAAACAAGTTTGATGATACTGAAA
52 L1 R		...C.....CTC...T..C..A..G..G.....C..C..C.....
52 L1 wt	(401)	CCAGTAACAAATATGCTGGTAAACCTGGTATAGATAATAGGGAATGTTTA
52 L1 R		..TC.....G..C.....G..A.....C.....C..A.....G
52 L1 wt	(451)	TCTATGGATTATAAGCAGACTCAGTTATGCATTTTAGGATGCAAACCTCC
52 L1 R	C..C.....A.....A..G..T..C..G..T..T..G..A..
52 L1 wt	(501)	TATAGGTGAACATTGGGGTAAGGGAACCCCTTGTAATAATAATTCAGGAA
52 L1 R		A..C.....C.....T..T..A.....C..C..C..T..T.
52 L1 wt	(551)	ATCCTGGGGATTGTCTCTCCCCTACAGCTCATTAACAGTGAATACAGGAT
52 L1 R		.C..A..T..C.....A..AT.G..AT.G..C...TCC..C..C..A..C
52 L1 wt	(601)	GGGGACATGGTAGATACAGGATTTGGTTGCATGGATTTTAATACCTTGCA
52 L1 R		..T.....C..C..T..T..C.....T.....C..C..C.....
52 L1 wt	(651)	AGCTAGTAAAAGTGATGTGCCCATTGATATATGTAGCAGTGTATGTAAGT
52 L1 R	TC...GTCC..C..C..A..C..C..C...TC.TC...C.....
52 L1 wt	(701)	ATCCAGATTATTTGCAAATGGCTAGCGAGCCATATGGTGACAGTTTGTTT
52 L1 R		.C.....C..C.....TCT..A.....C.....TCC.....
52 L1 wt	(751)	TTTTTCTTAGACGTGAGCAAATGTTTGTAGACACTTTTTTAATAGGGC
52 L1 R		..C..CT.G...A..A..A.....C..C.....C..C..C..A..
52 L1 wt	(801)	CGGTACCTTAGGTGACCCTGTGCCAGGTGATTTATATATACAAGGGTCTA
52 L1 R		T.....G.....A..T.....C..G..C..C.....T..C.
52 L1 wt	(851)	ACTCTGGCAATACTGCCACTGTACAAAGCAGTGCTTTTTTTCCTACTCCT
52 L1 R	T..C.....T.....C...TC.TC.....C..C..A.....A

52 L1 wt	(901)	AGTGGTTCTATGGTAACCTCAGAATCCCAATTATTTAATAAACCGTACTG
52 L1 R		TC.....C.....C.....C.....G..C..C..G..A.....
52 L1 wt	(951)	GTTACAACGTGCGCAGGGCCACAATAATGGCATATGTTGGGGCAATCAGT
52 L1 R		...G...A..A..T..A..T.....C..C..T..C.....T..C..A..
52 L1 wt	(1001)	TGTTTGTCTACAGTTGTGGATACCACTCGTAGCACTAACATGACTTTATGT
52 L1 R	C.....C..C..C..C..T...A..ATCT.....C..G...
52 L1 wt	(1051)	GCTGAGGTTAAAAAGGAAAGCACATATAAAAAATGAAAATTTTAAGGAATA
52 L1 R	A..C..G.....TC...C..C..G..C.....C..C.....
52 L1 wt	(1101)	CCTTCGTCATGGCGAGGAATTTGATTTACAATTTATTTTCAATTGTGCA
52 L1 R		.T.GA.A..C..T..A.....C..C..G.....C..C..C.....T..
52 L1 wt	(1151)	AAATTACATTAACAGCTGATGTTATGACATACATTTCATAAGATGGATGCC
52 L1 R		.G..C..C..G..C.....C..C.....T.....C..C.....C..T
52 L1 wt	(1201)	ACTATTTTAGAGGACTGGCAATTTGGCCTTACCCACCACCGTCTGCATC
52 L1 R	C..G..A.....C..TT.G..T.....A..C..T..
52 L1 wt	(1251)	TTTGGAGGACACATACAGATTTGTCACTTCTACTGCTATAACTTGTCAAA
52 L1 R		C.....A.....T.....C.....C.....C..C.....
52 L1 wt	(1301)	AAAACACGCCACCTAAAGGAAAGGAAGATCCTTTAAAGGACTATATGTTT
52 L1 R		.G.....T.....A..G..T.....C..A..G.....C.....C
52 L1 wt	(1351)	TGGGAGGTGGATTTAAAGAAAAGTTTCTGCAGATTTAGATCAGTTTCC
52 L1 R	A..C..C..G..G.....C.....T..C..G..C..A..C..
52 L1 wt	(1401)	TTTAGGTAGGAAGTTTTTGTACAGGCAGGGCTACAGGCTAGGCCCAAAC
52 L1 R		A..G.....A.....C.....G..A..T..TT.G..A.....A..A..GT
52 L1 wt	(1451)	TAAAACGCCCTGCATCATCGGCCCCACGTACCTCCACAAAGAAGAAAAG
52 L1 R		.G..GA.A..A..TAGC..T..T...A.A..T.....C.....G...
52 L1 wt	(1501)	GTAAAAGGTAA (SEQ ID NO:3)
52 L1 R		..C..G..A...(SEQ ID NO:1)

Figure 2. HPV 52 L1 R Nucleotide and Amino Acid Sequences.

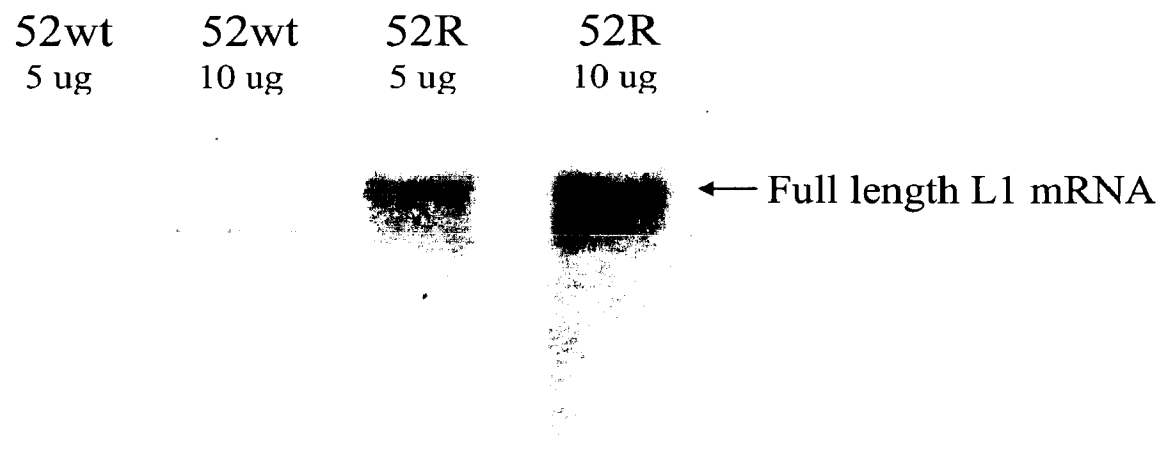
	M	S	V	W	R	P	S	E	A	T	V	Y	L	P	P	V	P
1	ATGTCCG	TCT	GGAGAC	CATC	CGAAGC	TACT	GTCTACT	TGC	CACCAG	TTC							
	TACAGGC	CAG	CCTCTG	G	TAG	GCTTCG	ATGA	CAGATGA	ACG	GTGGTCA	AGG						
	G	S	K	V	V	S	T	D	E	Y	V	S	R	T	S	I	Y
51	AGTCTCT	AAG	GTTGTCT	CTA	CCGACGA	AATA	CGTCTCC	CAGA	ACCTCC	ATCT							
	TCAGAGAT	TC	CAACAG	AGAT	GGCTGCT	TAT	GCAGAGG	TCT	TGGAGG	TAGA							
	Y	Y	A	G	S	S	R	L	L	T	V	G	H	P	Y	F	
101	ACTACTAC	G	TGGTTC	CCTCT	AGATTGT	TGA	CTGTTCG	G	TCA	CTTC							
	TGATGAT	GCG	ACCAAGG	GAGA	TCTAACA	ACT	GACAGCC	AGT	GGGTATG	AAAG							
	S	I	K	N	T	S	S	G	N	G	K	K	V	L	V	P	K
151	TCTATCA	AAGA	ACACCTC	CCTC	CGGTAAC	CGGT	AAGAAGG	TCT	TGGTTCC	AAAA							
	AGATAGT	TCT	TGTGGAG	GAG	GCCATTG	CCA	TTCTTCC	CAGA	ACCAAGG	TTT							
	V	S	G	L	Q	Y	R	V	F	R	I	K	L	P	D	P	N
201	GGTCTCT	G	TTGCAAT	AACA	GAGTCTT	CAG	AATCAAG	TG	CCAGACCC	AA							
	CCAGAGAC	CA	AACGTT	ATGT	CTCAGAA	AGTC	TTAGTTCA	AC	GGTCTGG	GTT							
	K	F	G	F	P	D	T	S	F	Y	N	P	E	T	Q	R	
251	ACAAGTT	CGG	TTTCCC	CAGAC	ACTAGTT	TTCT	ACAACCC	CAGA	AACTCAA	AGA							
	TGTTCAAG	CC	AAAGGG	TCTG	TGATCAA	AGA	TGTTGGG	TCT	TTGAGTT	TTCT							
	L	V	W	A	C	T	G	L	E	I	G	R	G	Q	P	L	G
301	TTGGTCT	G	CTTGTAC	TGG	TTTGGAA	ATC	GGTAGAG	GTC	AACCATT	G							
	AACCAGAC	CC	GAACATG	ACC	AAACCTT	TAG	CCATCTC	CAG	TTGGTA	ACCC							
	V	G	I	S	G	H	P	L	L	N	K	F	D	D	T	E	T
351	TGTCGGT	ATC	TCTGGTC	ACC	CATTGTT	GAA	CAAGTTC	GAC	GACACTG	AAA							
	ACAGCCAT	AG	AGACCAG	TGG	GTAACA	ACTT	GTTCAAG	CTG	CTGTGAC	TTT							
	S	N	K	Y	A	G	K	P	G	I	D	N	R	E	C	L	
401	CCTCTAA	CAA	GTACGCT	G	AAGCCAG	GTA	TCGATAA	CAG	AGAATGT	TTTG							
	GGAGATT	GTT	CATGCG	ACCA	TTCCGT	CCAT	AGCTATT	GTC	TCTTACA	AAAC							
	S	M	D	Y	K	Q	T	Q	L	C	I	L	G	C	K	P	P
451	TCTATGG	ACT	ACAAGCA	AAAC	TCAATTG	TGT	ATCTTGG	GTT	GTAAGCC	ACC							
	AGATACCT	G	TGTTTCG	TTG	AGTTAAC	ACA	TAGAACC	CAA	CATTCCG	TGG							
	I	G	E	H	W	G	K	G	T	P	C	N	N	N	S	G	N
501	AATCGGT	GAA	CACTGGG	GTA	AGGGTAC	TCC	ATGTAACA	CAAC	AACTCTG	GTA							
	TTAGCCAC	TT	GTGACCC	CCAT	TCCCATG	AGG	TACATTG	TG	TTGAGAC	CAT							
	P	G	D	C	P	P	L	Q	L	I	N	S	V	I	Q	D	
551	ACCCAGG	TGA	CTGTCC	ACCA	TTGCAAT	TGA	TCAACTC	CGT	CATCCA	AGAC							
	TGGGTCC	ACT	GACAGGT	G	AACGTTA	ACT	AGTTGAG	GCA	GTAGGTT	CTG							
	G	D	M	V	D	T	G	F	G	C	M	D	F	N	T	L	Q
601	GGTGAC	ATGG	TCGACAC	TGG	TTTCGGT	TGT	ATGGACT	TCA	ACACCTT	GCA							
	CCACTGT	ACC	AGCTGTG	ACC	AAAGCCA	ACA	TACCTGA	AGT	TGTGGA	ACGT							
	A	S	K	S	D	V	P	I	D	I	C	S	S	V	C	K	Y
651	AGCTTCT	AAG	TCCGACG	TCC	CAATCGA	CAT	CTGTTCC	TCT	GTCTGTA	AGT							
	TCGAAG	ATTC	AGGCTGC	AGG	GTTAGCT	GTA	GACAAGG	GAGA	CAGACAT	TCA							
	P	D	Y	L	Q	M	A	S	E	P	Y	G	D	S	L	F	
701	ACCCAG	ACTA	CTTGCAA	ATG	GCTTCTG	AAC	CATACGG	TGA	CTCCTTG	TTC							
	TGGGTCT	GAT	GAACGTT	TAC	CGAAGAC	TTG	GTATGCC	ACT	GAGGAAC	AAG							
	F	F	L	R	R	E	Q	M	F	V	R	H	F	F	N	R	A
751	TTCTTCT	TGA	GAAGAGA	ACA	AATGTT	CGTC	AGACACT	TCT	TCAACAG	AGC							

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      AAGAAGAACT CTTCTCTTGT TTACAAGCAG TCTGTGAAGA AGTTGTCTCG
      G T L G D P V P G D L Y I Q G S N
801  TGGTACCTTG GGTGACCCAG TTCCAGGTGA CTTGTACATC CAAGGTTCCA
      ACCATGGAAC CCACTGGGTC AAGGTCCACT GAACATGTAG GTTCCAAGGT
      S G N T A T V Q S S A F F P T P
851  ACTCTGGTAA CACTGCTACT GTCCAATCCT CTGCTTTCTT CCAACTCCA
      TGAGACCATT GTGACGATGA CAGGTTAGGA GACGAAAGAA GGGTTGAGGT
      S G S M V T S E S Q L F N K P Y W
901  TCTGGTTCCA TGGTCACCTC CGAATCCCAA TTGTTCAACA AGCCATACTG
      AGACCAAGGT ACCAGTGGAG GCTTAGGGTT AACAAGTTGT TCGGTATGAC
      L Q R A Q G H N N G I C W G N Q L
951  GTTGCAAAGA GCTCAAGGTC ACAACAACGG TATCTGTTGG GGTAACCAAT
      CAACGTTTCT CGAGTTCCAG TGTTGTTGCC ATAGACAACC CCATTGGTTA
      F V T V V D T T R S T N M T L C
1001 TGTTCGTCAC CGTCGTCGAC ACTACTAGAT CTACTAACAT GACCTTGTGT
      ACAAGCAGTG GCAGCAGCTG TGATGATCTA GATGATTGTA CTGGAACACA
      A E V K K E S T Y K N E N F K E Y
1051 GCTGAAGTCA AGAAGGAATC CACCTACAAG AACGAAAAC TCAAGGAATA
      CGACTTCAGT TCTTCCTTAG GTGGATGTTT TTGCTTTTGA AGTTCCTTAT
      L R H G E E F D L Q F I F Q L C K
1101 CTTGAGACAC GGTGAAGAAT TCGACTTGCA ATTCATCTTC CAATTGTGTA
      GAACTCTGTG CCACTTCTTA AGCTGAACGT TAAGTAGAAG GTTAACACAT
      I T L T A D V M T Y I H K M D A
1151 AGATCACCTT GACCGCTGAC GTCATGACTT ACATCCACAA GATGGACGCT
      TCTAGTGGA CTGGCGACTG CAGTACTGAA TGTAGGTGTT CTACCTGCGA
      T I L E D W Q F G L T P P P S A S
1201 ACTATCTTGG AAGACTGGCA ATTCGGTTTG ACTCCACCAC CATCCGCTTC
      TGATAGAACC TTCTGACCGT TAAGCCAAAC TGAGGTGGTG GTAGGCGAAG
      L E D T Y R F V T S T A I T C Q K
1251 CTTGGAAGAC ACTTACAGAT TCGTCACTTC CACTGCTATC ACCTGTCAAA
      GAACCTTCTG TGAATGTCTA AGCAGTGAAG GTGACGATAG TGGACAGTTT
      N T P P K G K E D P L K D Y M F
1301 AGAACACTCC ACCAAAGGGT AAGGAAGACC CATTGAAGGA CTACATGTTT
      TCTTGTGAGG TGGTTTCCCA TTCCTTCTGG GTAACCTCCT GATGTACAAG
      W E V D L K E K F S A D L D Q F P
1351 TGGGAAGTCG ACTTGAAGGA AAAGTTCTCT GCTGACTTGG ACCAATTCCC
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      L G R K F L L Q A G L Q A R P K L
1401 ATTGGGTTAGA AAGTTCTTGT TGCAAGCTGG TTTGCAAGCT AGACCAAAGT
      TAACCCATCT TTCAAGAACA ACGTTCGACC AAACGTTCTGA TCTGGTTTCA
      K R P A S S A P R T S T K K K K
1451 TGAAGAGACC AGCTAGCTCT GCTCCAAGAA CTTCCACCAA GAAGAAGAAG
      ACTTCTCTGG TCGATCGAGA CGAGGTTCTT GAAGGTGGTT CTTCTTCTTC
      V K R * (SEQ ID NO:2)
1501 GTCAAGAGAT AA (SEQ ID NO:1)
      CAGTTCTCTA TT (SEQ ID NO:7)

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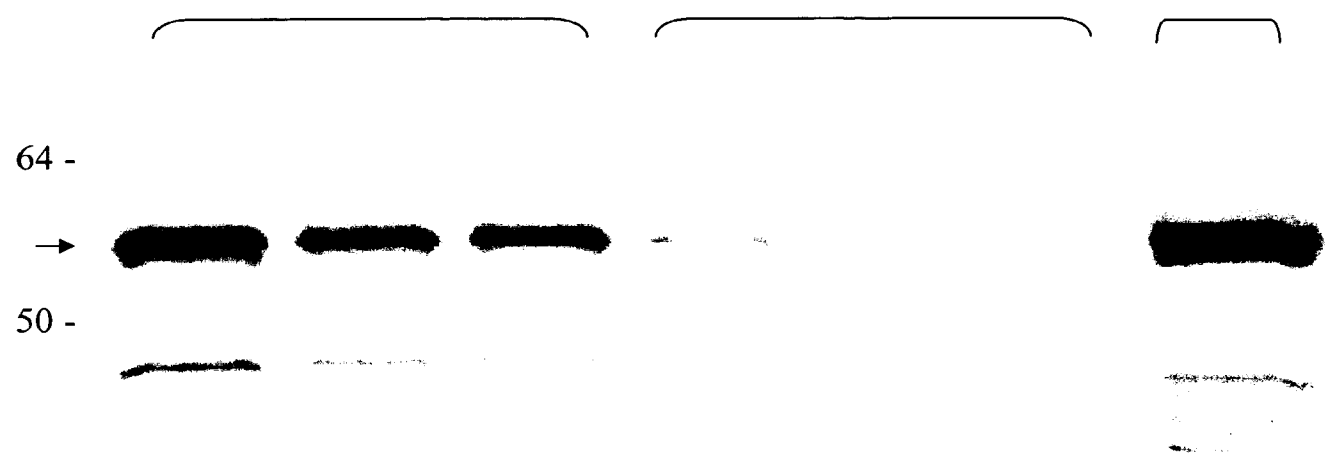
FIGURE 3. Expression of HPV 52 L1 wt and 52 L1 R Transcripts



21571PV.

vi 01 0

FIGURE 4. Western Blot Analysis of HPV 52 L1 Protein



21571PV

FIGURE 5. Transmission EM of VLPs Composed of HPV 52 L1 R Protein Molecules

